AD_____

Award Number: DAMD17-01-1-0773

TITLE: Non-invasive Gene Therapy of Experimental Parkinson's Disease

PRINCIPAL INVESTIGATOR: William M. Pardridge, M.D.

CONTRACTING ORGANIZATION: The University of California, Los Angeles
Los Angeles, California 90024-1406

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual (27 Aug 01 - 26 Aug 02)

4. TITLE AND SUBTITLE
Non-invasive Gene Therapy of Experimental Parkinson's Disease

5. FUNDING NUMBERS
DAMD17-01-1-0773

6. AUTHOR(S)
William M. Pardridge, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California, Los Angeles
Los Angeles, California 90024-1406

E-Mail: wpardridge@mednet.ucla.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The present research has developed a non-viral gene targeting technology, whereby the effects of a neurotoxin on the brain can be reversed shortly after the intravenous injection of a therapeutic gene medicine without the use of viral vectors. The brain gene targeting technology developed in this work creates an "artificial virus" which is comprised of non-immunogenic lipids and proteins, wherein the therapeutic gene is packaged in the interior of the gene delivery vehicle, which is called a pegylated immunoliposome (PIL). The PIL carrying the gene is a 85 nm "stealth" nanocontainer, which is relatively invisible to the body's reticuloendothelial system, which normally removes nanocontainers from the blood. The surface of the nanocontainer is studded with a receptor-specific monoclonal antibody (MAb). This MAb acts as a moleculr Trojan horse, and triggers the transport of the stealth nanocontainer across the 2 biologicial membrane barriers that separate the blood from the interior of brain cells. These barriers are the brain microvascular endothelial wall, which forms the blood-brain barrier in vivo, and the brain cell plasma membrane. Both barriers express the transferrin receptor, and the anti-receptor MAb enables the PIL to cross the membrane barriers via normal physiological transport processes usually used for endogeneous ligands such as transferrin. With this approach non-viral, non-invasive gene therapy of the brain is now possible.

14. SUBJECT TERMS	15. NUMBER OF PAGES
neurotixin, brain gene therapy, non-viral gene transfer	43
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Neurotoxins can cause serious derangements in brain biochemistry that can comprimise the cognitive and motor function of the individual. In the present studies an animal model of neurotoxin exposure is used, wherein 6-hyrdoxy dopamine is injected into the brain of rats, followed approximately 4 weeks later by a biochemical picture resembling Parkinson's disease (PD). On the side of the brain where the neurotoxin is injected, there is a 90% reduction in the level of a key enzyme, tyrosine hydroxylase (TH), that is a rate-limiting enzyme involved in dopamine production. Dopamine is the neurotransmitter that is deficient in PD. One way that brain TH levels can be restored in conditions such as PD is through gene therapy, wherein the TH gene is given to the individual afflicted with PD. However, with the conventional approach to gene therapy of the brain, there are two serious problems. First, virtually all present-day approaches use viral vectors to carry the gene to brain cells. However, these viral vectors are either highly inflammatory (such as adenovirus or herpes simplex virus) or stably alter the host genome in a random way (retrovirus, adeno-associated virus). These viruses do not enter the brain from blood, because they do not cross the blood-brain barrier (BBB). This creates the second problem with present-day approaches to gene therapy, which is the viral vector is administered to the brain by craniotomy and drilling a hole in the head. However, this only distributes the virus to a tiny region of the brain at the tip of the injection needle. What is needed is a non-invasive, non-viral form of brain gene therapy wherein the therapeutic gene can be administered intravenously without viral vectors followed by widespread expression of the exogenous gene throughout the brain. This is the goal of the present research.

The present research uses an "artificial virus" brain gene targeting technology which is comprised of non-immunogenic lipids and proteins, wherein the therapeutic gene is packaged within the interior of the gene delivery vehicle, which is called a pegylated immunoliposome (PIL). The PIL carrying the gene is an 85 nm "stealth" nano-container, which is relatively invisible to the body's reticuloendothelial system, that normally removes nano-containers from the blood. This stealth effect is created by conjugating approximately 2000 strands of 2000 Dalton polyethylene glycol (PEG) to the surface of the liposome carrying the gene inside. Approximately 1-2% of the tips of the PEG strands are studded with receptor-specific monoclonal antibodies (MAb). This MAb is a targeting ligand and acts as a molecular Trojan horse, which triggers the transport of the stealth nano-container across the two biological membrane barriers which separate the blood from the interior of brain cells: the brain microvascular endothelial wall, which forms the blood-brain barrier (BBB) in vivo, and the brain cell plasma membrane (BCM). Both the BBB and the BCM express a targeted receptor, in this case, the transferrin receptor (TfR), and the anti-TfR MAb enables the PIL to cross the membrane barriers via normal physiological transport processes which are usually used for endogenous ligands such as transferrin. With this approach, non-viral gene therapy, non-invasive gene therapy of the brain is now possible.

The TH expression plasmid is encapsulated in the interior of the 85 nm PIL which is targeted to rat brain with the OX26 murine MAb to the rat TfR. The TfRMAb-PIL carrying the plasmid DNA is injected intravenously in rats at a dose of 10 μg plasmid DNA per adult rat. These rats all have drug-confirmed experimental PD, owing to the intracerebral injection of the 6-hydroxydopamine neurotoxin into the brain four weeks earlier. The goal is to normalize the striatal TH activity based on both brain biochemistry assays, immunocytochemistry assays, and pharmacologic behavioral testing.

BODY

The results of the past study period are described in the attached manuscript, which is Appendix 1. Initially, we evaluated four TH expression plasmids, designated clones 877,878, 883, and 908, as shown in Figure 1 of Appendix 1. The measurement of TH gene expression in tissue culture with these four plasmids showed that the optimal plasmid was clone 877, which is derived from the pGL2 expression vector. The TH gene is driven by the SV40 promoter and is followed in the 3'-untranslated region (UTR) with a cis element from the Glut1 glucose transporter mRNA. This cis element stabilizes the expressed mRNA. The hypothetical structure of the PIL gene delivery system is shown in Figure 2A of Appendix 1, and this structure is confirmed by electron microscopy as shown in Figure 2B of Appendix 1.

We packaged the encapsulated clone 877 TH expression plasmid in a PIL targeted to either rat glioma cells in tissue culture using OX26 murine MAb to the rat TfR, or to human U87 glioma cells in tissue culture with the murine 8314 MAb to the human insulin receptor. These studies showed that the PIL gene delivery system enabled levels of TH gene expression in cultured cells that were comparable to either lipofectamine or to viral vector systems (Figure 3 of Appendix 1).

We next measured TH enzyme activity in the striatum and cortex of the neurotoxin lesioned animals at three days after a single intravenous injection of the gene therapeutic. The biochemistry results are shown in Table 2 of Appendix 1, and indicate the neurotoxin results in a greater than 90% reduction in ipsilateral striatal TH enzyme activity. Figure 4 of Appendix 1 shows that the neurotoxin causes nearly a complete loss of striatal immunoreactive TH. Conversely, with the intravenous gene therapy (single dose format) we were able to completely normalize both the TH enzyme activity (Table 2 of Appendix 1) and the level of immunoreactive TH based on immunocytochemistry (Figure 4 of Appendix 1).

The single intravenous injection of the TH gene therapy caused a 70% improvement in apomorphine induced rotation behavior in the lesioned and treated animals, as shown in Figure 5 of Appendix 1.

When the lesioned animals were treated with the gene therapy in an identical formulation, except the non-specific isotype control antibody replaced the transferrin receptor MAb, then we found there was no beneficial effect of the gene therapy. There was no effect on TH enzyme activity in brain. There was no increase in striatal immunoreactive TH based on immunocytochemistry, and there was no significant change in the rotation behavior induced by apomorphine. This side-by-side comparison of the receptor-specific MAb and the non-specific isotype control indicates the beneficial effects of the gene therapy is due strictly to the targeting specificity of the molecular Trojan horse attached to the PIL formulation, as depicted in Figure 2 of Appendix 1.

KEY RESEARCH ACCOMPLISHMENTS

The following publications were produced in the last reporting year:

- Shi, N., Zhang, Y., Boado, R.J., Zhu, C., and Pardridge, W.M. (2001): Brain-specific expression of an exogenous gene following intravenous adminstration. Proc. Natl. Acad. Sci.

U.S.A., 98: 12754-12759. (Reviewed in *Nature Reviews-Neuroscience* 2: 852-853, December, 2001.).....This shows that it is possible to have widespread expression of an exogenous gene throughout the brain following a simple intravenous injection of a non-viral gene delivery system.

- Pardridge, W.M. (2002): Drug and gene targeting to the brain with molecular Trojan horses. Nature Reviews-Drug Discovery, 1: 131-139.....This article reviews the concept of the use of molecular Trojan horses to ferry across the blood-brain-barrier therapeutic genes.

- Zhang, Y., Zhu, C., and Pardridge, W.M. (2002): Antisense gene therapy of brain cancer with an artificial virus gene delivery system. Mol. Ther., 6: 67-72.....This study shows that it is possible to achieve 100% increase in survival time in mice with experimental human brain cancer using the non-invasive, non-viral gene delivery system.

- Zhang, Y., Boado, R.J., and Pardridge, W.M. (2002): Marked enhancement in gene expression by targeting the human insulin receptor. J. Gene Med., in press.....This study measures luciferase gene expression in cultured cells and shows that targeting the human insulin receptor results in 10-fold higher levels of gene expression as compared to targeting other receptors such as the transferrin receptor or the epidermal growth factor receptor.

- Zhang, Y., Calon, F., Zhu, C., Boado, R.J., and Pardridge, W.M. (2002): Intravenous non-viral gene therapy causes complete normalization of striatal tyrosine hydroxylase and reversal of motor impariment in experimental Parkinsonism. Submitted for publication.....This paper shows that it is possible to achieve complete normalization of brain tyrosine hydroxylase in neurotoxin-lesioned rat brain with non-viral, non-invasive gene therapy of the brain (Appendix 1).

REPORTED OUTCOMES

(1) Manuscripts: listed above in key research accomplishments.

(2) Plasmids developed: several tyrosine hydroxylase expression plasmids were produced as described in Appendix 1, Figure 1.

CONCLUSIONS

This research has shown the injection of a neurotoxin into the median forebrain bundle of rat brain causes a nearly 90% decrease in tyrosine hydroxylase enzyme activity and immunoreactive tyrosine hydroxylase in the ipsilateral striatum, which is the part of the brain that is deranged in Parkinson's disease. This research was able to show, for the first time, that it is possible to completely normalize striatal tyrosine hydroxylase activity in the toxin-treated animals with a single intravenous injection of a non-viral tyrosine hydroxylase expression plasmid packaged in the interior of a pegylated immunoliposome (PIL). The PIL gene targeting technology is the first non-viral gene transfer technology developed that allows for widespread

expression of an exogenous gene in a remote target organ following a simple intravenous injection. This research has medical implications, and suggests that a new approach is being developed for the gene therapy of Parkinson's disease that does not require either the use of viral vectors or craniotomy. The potential applications of this gene targeting technology extend beyond the realm of Parkinson's disease and could potentially be used for the gene therapy of a wide variety of brain disorders.

APPENDICES

(1) Zhang, Y., Calon, F., Zhu, C., Boado, R.J., and Pardridge, W.M. (2002): Intravenous non-viral gene therapy causes complete normalization of striatal tyrosine hydroxylase and reversal of motor impariment in experimental Parkinsonism. Submitted for publication

Appendix 1

Intravenous Non-Viral Gene Therapy Causes Complete Normalization of Striatal Tyrosine Hydroxylase and Reversal of Motor Impairment in Experimental Parkinsonism

Yun Zhang
Frederic Calon
Chunni Zhu
Ruben J. Boado
William M. Pardridge

Department of Medicine
UCLA School of Medicine
Los Angeles, CA 90024

Pages: 36
Words: Abstract (243); Introduction (550); Discussion (1461)
Figures: 5 **Tables**: 2

Running Title: Non-invasive, non-viral gene therapy of the brain

Acknowledgments: This work was supported by a grant from the Neurotoxin Exposure Treatment Research Program of the U.S. Department of Defense. F.C. was supported by the Canadian Institutes of Health Research.

Correspondence: W. M. Pardridge, UCLA Warren Hall 13-164, 900 Veteran Avenue, Los Angeles, CA 90024. Phone: (310) 825-8858; Fax: (310) 206-5163; Email: wpardridge@mednet.ucla.edu

Keywords: gene therapy, blood-brain barrier, transferrin receptor, tyrosine hydroxylase, Parkinson's disease, liposomes

ABSTRACT

Brain gene targeting technology is used to normalize the tyrosine hydroxylase (TH) activity in the striatum of rats with the 6-hydroxydopamine model of experimental Parkinson's disease. The TH expression plasmid is encapsulated in the interior of an 85 nm pegylated immunoliposome (PIL) that is targeted with either the OX26 murine monoclonal antibody (MAb) to the rat transferrin receptor (TfR) or the mouse IgG2a isotype control antibody. The TfRMAb-PIL, or the mIgG2a-PIL, is injected intravenously at a dose of 10 ug plasmid DNA/adult rat. The TfRMAb-PIL, but not the mIgG2a-PIL, enters brain via the trans-vascular route. The targeting TfRMAb enables the nanocontainer carrying the gene to undergo both receptor-mediated transcytosis across the blood-brain barrier (BBB) and receptor-mediated endocytosis into neurons behind the BBB by accessing the TfR. With this approach, the striatal TH activity ipsilateral to the intra-cerebral injection of the neurotoxin was normalized and increased from 738 ± 179 pmol/hr/mg_p to 5486 ± 899 pmol/hr/mg_p. The TH enzyme activity measurements were corroborated by TH immunocytochemistry, which showed the entire striatum was immunoreactive for TH following intravenous gene therapy. The normalization of striatal biochemistry was associated with a reversal of apomorphine-induced rotation behavior. Lesioned animals treated with the apomorphine exhibited 20 ± 5 and 6 ± 2 rotations per min after intravenous administration of the TH plasmid encapsulated in the mIgG2a-PIL and the TfRMAb-PIL, respectively. These studies demonstrate it is possible to completely normalize brain enzyme activity with intravenous gene therapy without viral vectors.

Gene therapy of Parkinson's disease (PD) aims to prevent striatal neurodegeneration and restore striatal tyrosine hydroxylase (TH) enzyme activity (Mouradian and Chase, 1997; Mandel et al, 1999). Striatal TH enzyme activity is partially restored in experimental PD by intra-cerebral injection of viral vectors encoding the TH gene (During et al, 1994; Kaplitt et al, 1994; Mandel et al,1998). However, viral vectors must be administered intra-cerebrally via craniotomy. The viruses cannot be administered intravenously because the viruses do not cross the brain capillary endothelial wall, which forms the blood-brain barrier (BBB) in vivo. The intra-cerebral injection of the viral vector, however, only leads to the transduction of a small part of the striatum at the tip of the injection needle. Higher fractions of the striatum may be transduced by multiple injections of high viral titers in either rat (Leone et al, 2000; Kirik et al, 2002) or primate brain (Kordower et al, 2000). However, the human brain is a 1000- and 10-fold larger than the rat or primate brain, respectively. Therefore, the transduction of a significant portion of the human striatum following intra-cerebral administration of therapeutic genes may be difficult.

The entire volume of the striatum may be transduced following trans-vascular delivery of the therapeutic gene subsequent to intravenous administration of the gene. The trans-vascular route to the striatum would be possible with the development of a gene targeting system that is capable of transport across the BBB. Recently, gene targeting technology has been developed that enables the widespread expression within the brain of non-viral plasmid DNA following intravenous administration (Shi et al, 2000; 2001a,b). The plasmid DNA encoding the therapeutic gene is encapsulated in the interior of a nano-container comprised of a pegylated immunoliposome (PIL), which is targeted across both the BBB and the neuronal cell membrane by receptor-specific monoclonal antibodies (MAb). Delivery of the therapeutic gene across the

BBB has the potential to transduce virtually every cell of the striatum, because every neuron in the brain is perfused by its own blood vessel. With the PIL gene targeting system, it is possible to deliver an exogenous gene throughout the entire central nervous system (CNS) following intravenous administration. Gene expression can be restricted to the brain with the use of brain-specific promoters (Shi et al, 2001a). Prior work with this gene targeting technology yielded a therapeutic result in an experimental brain cancer model with 100% increase in the survival time of the animals treated with intravenous antisense gene therapy (Zhang et al, 2002a). The present studies attempt to normalize the striatal TH activity and motor impairment in the 6-hydroxy dopamine model of experimental PD in the rat. The full length rat TH cDNA is incorporated in a pGL2-derived expression plasmid that is driven by the SV40 promoter and contains a cis stabilizing element in the 3'-untranslated region (UTR) of the mRNA. The plasmid DNA is encapsulated in the interior of the PIL, which is targeted to brain with the murine OX26 MAb to the rat transferrin receptor (TfR). Owing to the expression of the TfR on both the rodent and human BBB (Jefferies et al, 1984; Pardridge et al, 1987), and the neuronal plasma membrane (Mash et al, 1991), the TfRMAb-targeted PIL is able to undergo receptor-mediated transcytosis across the BBB followed by receptor-mediated endocytosis into neurons behind the BBB.

MATERIALS AND METHODS

Materials. POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine) and DDAB (didodecyldimethylammonium bromide) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL). Distearoylphosphatidylethanolamine (DSPE)-PEG^{2000} was obtained from Shearwater Polymers (Huntsville, AL), where PEG^{2000} is 2000 Dalton polyethyleneglycol. DSPE-PEG^{2000}-maleimide (MAL) was custom synthesized by Shearwater Polymers. [α-^{32}P]dCTP (3000 Ci/mmol) and [3,5-^{3}H]-L-tyrosine (51.5 Ci/mmol) were from NEN Life Science Product Inc. (Boston, MA). N-succinimidyl[2,3-^{3}H]propionate (^{3}H-NSP, 101 Ci/mmol) and Protein G Sepharose CL-4B were purchased from Amersham-Pharmacia Biotech (Arlington Heights, IL). The nick translation system was from Life Technologies Inc. (Rockville, MA). The 6-hydroxydopomine (6-OHDA), apomorphine, pargyline, catalase, (6R)-5,6,7,8-tetrahydrobiopterin (BH4), β-NADPH, L-tyrosine, charcoal, the mouse MAb against rat tyrosine hydroxylase, horse serum, mouse IgG1 isotype control, and glycerol-gelatin were purchased from Sigma (St. Louis, MO); 2-iminothiolane (Traut's reagent) and bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co. (Rockford, IL). Mouse myeloma ascites containing mouse IgG2a (mIgG2a) isotype control was from Cappel Division of ICN Pharmaceuticals (Aurora, OH). The anti-transferrin receptor monoclonal antibody (TfRMAb) used in these studies is the murine OX26 MAb to the rat TfR, which is a mouse IgG2a. The OX26 MAb is specific for the rat TfR, and is not active in human cells. The anti-insulin receptor MAb used for gene targeting to human cells is the murine 83-14 MAb to the human insulin receptor (HIR). The TfRMAb, the HIRMAb, or the mIgG2a were individually purified with protein G affinity chromatography from hybridoma generated ascites. COS-1 cells were

obtained from the American Type Culture Collection (Rockford, MD). The biotinylated horse anti-mouse IgG, Vectastain ABC kit, 3-amino-9-ethylcarbazole (AEC) substrate kit for peroxidase kit and hematoxylin QS counterstain were purchased from Vector Laboratories (Burlingame, CA). Optimal cutting temperature compound (OCT; Tissue-Tec) was purchased from Sakura FineTek (Torrance, CA). Lipofectamine was obtained from Invitrogen (San Diego, CA).

Construction of tyrosine hydroxylase expression plasmids. The complete open reading frame (orf) of the rat (r) TH in the pBabe plasmid was obtained from Dr. Dale Bredesen (Buck Center, Novato, CA). (Anton et al, 1994). The rat TH orf was isolated by double digestion with DraI and Acc65I, which cleaved at sites located 26 nucleotides upstream and 13 nucleotides downstream of the rat TH orf, respectively. The ~1.5 kb rTH fragment was purified by gel electrophoresis followed by centrifugation with a Spin X filter unit. The DNA was blunt ended with Klenow DNA polymerase, and subcloned in Bluescript (pBS, Stratagene, San Diego, CA) at the EcoRV site to form a plasmid named pBS-rTH. The identity of rat TH and its orientation in pBS-rTH were determined by DNA sequencing using M13 forward and reverse primers. The rat TH cDNA was subcloned in the pGL2 promoter-derived mammalian expression vectors described previously and designated clones 734 and 753 (Dwyer et al. 1996). The pGL2 promoter plasmid was obtained from Promega (Madison, WI), and the pGL2-derived clones are driven by the SV40 promoter, and clone 753 contains a 200 nucleotide cis-element taken from nucleotides 2100-2300 of the bovine Glut1 mRNA 3'-untranslated region (UTR), which causes stabiliztion of the mRNA (Boado and Pardridge, 1998). The pBS-TH was linearized with NotI and blunt-ended with Klenow DNA polymerase. The 1.5 kb rat TH was released with HindIII

and purified by gel electrophoresis and Spin X centrifugation. In parallel, the luciferase reporter gene in clones 734 and 753 was deleted with HindIII and EcoNI (blunt) and purified. The rat TH DNA fragment was ligated into clones 734 and 753 with T4 DNA ligase and E. coli DH5α were transformed. Positive clones were investigated with restriction endonuclease mapping using PstI, which releases the TH insert, and DNA sequencing using the pGL2-1 sequencing primer (Promega). The pGL2-rTH expression vector that was derived from clone 734 was designated clone 878, and the pGL2-rTH expression vector containing the GLUT1 cis-stabilizing sequence, and derived from clone 753, was designated clone 877. The SV40-rTH expression cassettes were released from clones 877 and 878 and further subcloned in the pCEP4 expression vector to form clone 908 and 883, respectively. The pCEP4 vectors contain the Epstein-Barr virus replication origin (oriP) and nuclear antigen (EBNA-1), which enable extrachromosomal replication in human cells. Clones 877 and 878 were digested with XhoI, SalI and ScaI to release the ~2.9 kb SV40-rTH fragments. In parallel, the pCEP4 vector was digested with SalI/XhoI to release the CMV-cassette and purified as previously described (Boado and Pardridge, 1998). The SV40-rTH expression cassettes were ligated into pCEP4. Positive clones were identified by restriction endonuclease mapping with NruI and HindIII, and confirmed by DNA sequencing as previously described for GLUT1 reporter genes (Boado and Pardridge, 1998). All 4 TH expression plasmids (877, 878, 883, and 908) are driven by the SV40 promoter. Clones 877 and 908 contain the Glut1 3'-UTR cis stabilzing element, and clones 883 and 908 contain the EBNA-1/oriP cis/trans elements for extrachromasomal replication in huma cells. The pGL2-derived TH clones, 877 and 878, are approximately 6.0 kb in size, and the pCEP4-derived TH clones, 883 and 908, are approximately 11.0 kb in size (Boado and Pardridge, 1998). Maxi-prep DNA was purified and plasmid DNA was ^{32}P-labeled as described previously (Zhang et al, 2002b).

The expression of the TH gene in cell culture was first evaluated with Lipofectamine transfection of either C6 rat glial cells or COS-1 cells, which were cultivated in Dulbecco's Modified Eagle medium (DMEM) with 10% calf serum or DMEM with high glucose (4.5 g/L) and 10% fetal bovine serum (FBS), respectively. Plasmid DNA was amplified with the QIAfilter Plasmid Maxi-Prep kit (Qiagen) and COS-1 or C6 glial cells were transfected with Lipofectamine as described previously (Shusta et al, 2002). Cells were seeded on 60 or 100 mm dishes at a density of 80,000 cells/cm^2 and the Lipofectamine/plasmid DNA (10:1 wt/wt ratio) was added in medium without serum for 4 hours. The complex was then removed, and fresh medium with serum was added and the cells were incubated for 48 hours prior to extraction and measurement of TH enzyme activity as described below.

Pegylated liposome synthesis and plasmid DNA encapsulation. POPC (18.8 μmol), DDAB (0.4 μmol), DSPE-PEG 2000 (0.6 μmol), and DSPE-PEG 2000-maleimide (0.2 μmol) were dissolved in chloroform followed by evaporation, as described previously (Zhang et al, 2002b). The lipids were dispersed in 0.2 ml 0.05 M Tris-HCl buffer (pH = 7.0) and vortexed 1 min followed by 2 min of bath sonication. Supercoiled DNA was ^{32}P-labeled with [α-^{32}P]dCTP by nick translation as described previously (Shi et al, 2000). Unlabeled plasmid DNA (250 μg) and 1 μCi [^{32}P]-labeled plasmid DNA were added to the lipids. The dispersion was frozen in ethanol/dry ice for 5 min and thawed at room temperature for 25 min, and this freeze-thaw cycle was repeated 5 times to produce large vesicles with the DNA loosely entrapped inside. The large vesicles were converted into small 85 nm diameter lipsomes by extrusion. The liposome dispersion was diluted to a lipid concentration of 40 mM, followed by extrusion 5 times each

through two stacks each of 200-nm and 100-nm pore size polycarbonate membranes, by using a hand held LipoFast™-Basic extruder (Avestin, Ottawa, Canada), as described previously (Shi et al, 2001a). The mean vesicle diameters were determined by quasielastic light scattering by using a Microtrac Ultrafine Particle Analyzer (Leeds-Northrup, St. Petersburg, FL), as described previously (Huwyler et al, 1996).

Approximately half of the DNA is interiorized in the small liposomes and about half is exteriorized. The plasmid DNA absorbed to the exterior of the liposomes was quantitatively removed by nuclease digestion (Shi et al, 2000). For digestion of the unencapsulated DNA, 5 units of pancreatic endonuclease I and 5 units of exonuclease III were added in 5 mM $MgCl_2$ to the liposome/DNA mixture after extrusion (Monnard et al, 1997). After incubation at 37 C for 1 h, the reaction was stopped by adding 20 mM EDTA. The extent to which the nuclease digestion removed the exteriorized plasmid DNA was determined by agarose gel electrophoresis and ethidium bromide staining of aliquots taken before and after nuclease treatment, as described previously (Shi et al, 2000). The entrapped plasmid DNA is completely resistant to high local concentrations of nuclease. The DNA encapsulated within the pegylated liposome is designated pegylated liposome/DNA.

MAb conjugation to the pegylated liposome/DNA. The TfRMAb, the HIRMAb, or the mIgG2a were thiolated and individually conjugated to the MAL moiety of the pegylated liposome/DNA to produce a pegylated immunoliposome (PIL) with the desired receptor (R)-specificity. The PIL conjugated with the OX26 MAb is designated the TfRMAb-PIL and the PIL conjugated with the mIgG2a isotype control is designated the mIgG2a-PIL. Either MAb or mIgG2a was radiolabeled with ^{3}H-NSP as described previously (Pardridge et al, 1992). The

[^{3}H]MAb had a specific activity of > 0.11 μCi/μg and a trichloroacetic acid (TCA) precipitability of > 97 %. The MAb (3.0 mg, 20 nmol) was thiolated with a 40:1 molar excess of 2-iminothiolane (Traut's reagent), as described previously (Huwyler et al, 1996). The thiolated MAb , which contained a trace amount of [^{3}H] labeled MAb, was conjugated to the pegylated liposome overnight and unconjugated MAb (and oligonucleotides produced by nuclease treatment) were separated from the PIL by Sepharose CL-4B column chromatography as described previously (Shi et al, 2000). The number of MAb molecules conjugated per liposome was calculated from the total [^{3}H]-MAb cpm in the liposome pool and the specific activity of the labeled MAb, assuming 100,000 lipid molecules per liposome, as described previously (Zhang et al, 2002b). The average number of MAb molecules conjugated per liposome was 52 ± 8 (mean ± SD, n = 4 syntheses). The final percentage entrapment of 250 μg of plasmid DNA in the liposome preparation was computed from the ^{32}P radioactivity and was 35.6 ± 2.1 % (mean ± SD, n = 4 synthesis) or 89 ug of plasmid DNA

The PIL solution was sterilized for use in tissue culture by passage through a 0.22 μm Millex-GV filter (Millipore Co., Bedford, MA) and the PIL is not structurally altered by this filtration step (Zhang et al, 2002b).

Electron microscopy. IgG conjugated PIL carrying plasmid DNA was examined with a conjugate of 10 nm gold and a goat anti-mouse secondary antibody (Sigma G7652). A 5 μL aliquot of the 83-14-PIL (5 x 10^{12} liposome-conjugated MAb) was incubated with 72 μL IgG gold conjugate (1 x 10^{12} gold particles) for 1 hour in 0.05 M Tris buffered saline, pH=6.9, with 0.7% bovine serum albumin, 5% FBS, and 12% glycerol in a total volume of 125 uL. Gold conjugated secondary antibody bound to the PIL was separated from unbound gold conjugate

with a 0.7 x 10 cm column of Sepharose CL-4B (BioRad, Hercules, CA). An aliquot (10 uL) of the eluate was applied to formvar-coated 2000 mesh copper grids, washed twice with 0.05 M Tris/0.15 M NaCl/pH=7.4, counter-stained with 2% uranyl acetate for 1 min, and then examined directly with electron microscopy using a Jeol JEM-100CX II electron microscope at 80kV. Negatives, taken at a 29,000 magnification, were scanned and enlarged in Adobe Photoshop 5.5 on a G4 Power Macintosh.

TH gene expression in cultured brain cells with the PIL gene targeting system. Human U87 glioma cells or rat RG2 glioma cells were plated on 60-mm collagen-treated dishes with MEM or F12 Ham medium containing 10 % FBS, respectively. When the cells reached 60 % confluence, the medium was removed by aspiration, and 6 ml of fresh medium containing 10% FBS was added to the cells, followed by the addition of 142 ul of the HIRMAb-PIL carrying the 877 DNA (4 ug plasmid DNA/dish) or the TfRMAb-PIL carrying the 877 DNA (4 ug of plasmid DNA/dish). The cells were incubated for 2, 4 or 6 days with 3 dishes at each time point for measurement of TH enzyme activity.

6-Hydroxydopamine model. Adult male Sprague-Dawley rats (supplied by Harlan Breeders, Indianapolis, IN) weighing 200-250 g were anesthetized with ketamine (50 mg/kg) and xylazine (4mg/kg) intraperitoneally (ip). Animals received unilateral 6-OHDA injections into the right medial forebrain bundle (Armstrong et al, 2002; Meshul et al, 2002). Each animal received pargyline 30-60 min prior to surgery (50 mg/kg in normal saline, ip). Following pargyline administration, 4 ul of 2 ug/ul of 6-OHDA (prepared freshly in 0.2 ug/ul ascorbic acid) was injected over a 4-min period using a 10-ul Hamilton syringe with the following stereotaxic

coordinates: -4.4 mm anterior to bregma, -1.0 mm lateral to bregma, and 7.8 mm below the dura. The syringe needle was left in place for 2 min after the injection to allow for diffusion of the toxin. Three weeks following the lesion, rats were tested for apomorphine-induced contralateral turning using 0.5 mg/kg of apomorphine injected intraperitoneally. Full (360°), contralateral rotations only were counted over 20 min starting 5 min after apomorphine administration, and rats turning more than 80 times in 20 min, or 4 rotations per min (RPM), were treated 1 week later with TH gene therapy.

For each experiment, 16 successfully lesioned rats were divided into two groups: (1) control group: 8 rats received 10 ug/rat of clone 877 DNA encapsulated in mIgG2a-PIL; (2) treatment group: 8 rats received 10 ug/rat of clone 877 DNA encapsulated in TfRMAb-PIL. The PIL was administered via the femoral vein; 3 days later the rats were tested for apomorphine-induced rotation behavior and then sacrificed. In each group, 3 rats were used for tyrosine hydroxylase (TH) immunocytochemistry assay, and 5 rats were used for TH biochemistry assay.

Tyrosine hydroxylase (TH) assay. The TH activity assay was performed according to Reinhard et al (1986) and Horellou et a*l* (1989) with modifications. TH converts [3,5-^{3}H]-L-tyrosine to both [^{3}H]-water and L-Dopa in a 1:1 stoichiometric relationship, and the 2 metabolites are separated by charcoal, which selectively binds the amino acids. For cultured cells, at the end of the incubation, the growth medium was removed. The cells were washed three times with cold wash buffer (5 mM potassium phosphate buffer), and then 400 ul of sonication buffer (wash buffer with 0.2 % triton X-100) was added to each dish. The cells were collected, and after a short vortex, the cells were sonicated for 30 seconds with a Branson Sonifier Cell Disruptor Model 185. The cell homogenate was centrifuged at 10,000 *g* for 10 min at 4 C, and

the supernatant was removed for TH assays. For TH assays in rat organs, the liver, the frontal cortex, and the dorsal striatum in both lesioned (ipsilateral) and non-lesioned (contralateral) sides of brain were frozen in dry ice. The tissue was transferred to a chilled glass tissue grinder containing 0.5 ml of cold wash buffer, and was homogenized with 10-15 strokes at 4 C followed by centrifugation at 10,000 *g* for 20 min, and the supernatant was removed for TH assays.

The tissue supernatants (200ul) were added to 100 ul of assay buffer [0.5 mM NADPH, 1 mM BH_4, 2600 units of catalase, 20 uM $Fe(NH_4)_2(SO_4)_2$, 10 uM L-tyrosine, 40-50 uCi/ml of ^{3}H-L-tyrosine, 50 mM potassium phosphate] to start the incubation at 37 C for 45 min. The reaction was stopped by the addition of 1 ml of 7.5 % charcoal in 1.0 M HCl. The mixture was vortexed 2 seconds and centrifuged at 500 *g* for 10 min. The supernatant was counted for the radioactivity of the ^{3}H-water product with a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer. The supernatant radioactivity was measured in parallel with assays blanks, and all assay measurements were at least 10-fold above the assay blank. The protein concentration in the cell extract was determined with the BCA protein assay reagent. The CPM was converted to pmol of L-Dopa based on the [^{3}H]-tyrosine specific activity, and the results were expressed as pmol L-Dopa/hour/mg protein.

Immunocytochemistry. Tyrosine hydroxylase immunocytochemistry was performed with the avidin biotin (ABC) immunoperoxidase method (Vector Laboratories). Brains were removed immediately after sacrifice, cut into coronal slabs through the striatum, embedded in OCT medium and frozen in dry ice powder. Frozen sections (20 μm) of rat brain were cut on a Mikron HM505E cryostat, and were fixed in 4% paraformaldehyde for 20 minutes at 4°C. Endogenous peroxidase was blocked with 0.3% H_2O_2 in 0.3% horse serum/PBS for 30 minutes. Non-specific

binding of proteins was blocked with 10% horse serum in 0.1% Triton X-100/PBS for 30 minutes. Sections were then incubated in either mouse anti-TH MAb (0.2 μg/ml) or mouse IgG1 isotype control (0.2 μg/ml) overnight at 4°C. After wash in PBS, sections were incubated in biotinylated horse anti-mouse IgG for 30 minutes and then in ABC for 30 minutes. After development in AEC, sections were mounted with glycerol-gelatine with or without lightly counterstaining with hematoxylin.

Statistical analyses. Statistically significant differences in TH enzyme activity in different brain regions and treatment groups were determined an analysis of variance (ANOVA) with Bonferroni correction using program 7D of the BMDP Statistical Software package developed by the UCLA Biomedical Computing Series. A p value <0.05 was considered significant.

RESULTS

Evaluation of TH expression plasmid activity in COS cells and C6 glioma cells transfected with Lipofectamine

The 4 TH expression plasmids were combined with the lipofectamine and individually added to either COS-1 or C6 rat glioma cells, and TH enzyme activity was measured 48 hours later (Figure 1). A similar pattern of TH gene expression was observed in either cell line, although the TH enzyme activity in the COS-1 cells was 10-fold greater than in the C6 cells, consistent with the selective activation of genes driven by the SV40 promoter in COS-1 cells. In either cell line, the pGL2-derived plasmid, clone 877, produced the highest level of TH enzyme activity. Therefore, clone 877 was selected for subsequent encapsulation in the TfRMAb-PIL for vivo gene therapy in rats with the experimental PD.

Transfection of cultured cells with the PIL gene targeting system

The encapsulation of clone 877 plasmid DNA within the PIL gene targeting system is depicted in Figure 2A. The targeting MAb molecules tethered to the tips of the PEG strands on the PIL are visualized with electron microscopy using an anti-mouse secondary antibody conjugated with 10 nm gold (Figure 2B). Since the targeting MAb molecules are species-specific, the clone 877 plasmid DNA was targeted to human U87 glioma cells with the PIL conjugated with the 83-14 MAb to the human insulin receptor (HIR). Conversely, rat RG-2 glioma cells were transduced with the PIL conjugated with the OX26 murine MAb to the rat transferrin receptor (rTfR). Clone 877 plasmid DNA was encapsulated in either the TfRMAb-PIL or the HIRMAb-PIL and 4 ug plasmid DNA was applied to each 60 mm culture dish containing either RG2 or U87 cells, respectively. The level of TH enzyme activity in either the

U87 cells or the RG-2 cells increased with time of incubation and peaked at four days after the single application of the PIL formulation at zero time (Figure 3). The TH enzyme activity in the rat RG-2 cells targeted with the OX26-PIL system was comparable to the TH enzyme activity in C6 glioma cells targeted with lipofectamine (Figures 1 and 3). The TH enzyme activity in the human U87 glioma cells targeted with the HIRMAb-PIL delivery system was comparable to TH enzyme activity produced with lipofectamine in COS-1 cells (Figures 1 and 3).

TH enzyme activity in liver of control rats treated with intravenous gene therapy

The TfRMAb-PIL targeting of the TH gene was initially examined in a TfR-rich peripheral organ, liver, in control rats, so as to identify the time course of gene expression. Control rats were administered 10 ug/rat of clone 877 plasmid DNA encapsulated in the TfRMAb-PIL and hepatic TH enzyme activity was measured at 0, 1, 2, and 3 days after the single intravenous administration (Table 1). The TH activity in control rat liver increased > 30-fold above basal levels by 3 days after gene administration. Therefore, 72 hours was chosen as the time point for measuring brain TH activity after intravenous TH gene therapy in the rats with experimental PD.

TH enzyme activity and immunoreactive TH levels in rat brain in experimental Parkinsonism treated with intravenous TH gene therapy

Three weeks after the intra-cerebral injection of the 6-hydroxydopamine, rats were treated with apomorphine and rotation behavior was examined. Those rats that demonstrated contralateral rotation in response to apomorphine were selected for subsequent treatment with the PIL gene targeting system a week later. Rats with experimental PD were treated either with

clone 877 plasmid DNA encapsulated in the PIL targeted with the TfRMAb (designated TfRMAb-PIL) or clone 877 plasmid DNA encapsulated in PIL targeted with the mouse IgG2a isotype control antibody (designated mIgG2a-PIL). The plasmid DNA was injected intravenously at a dose of 10 ug DNA per rat, and animals were sacrificed 72 hours after the single intravenous injection for measurement of either TH enzyme activity in dorsal striatal or cortical homogenates or immunoreactive TH levels by immunocytochemistry. The TH enzyme activity in the dorsal striatum ipsilateral to the 6-hydroxydopamine injection was reduced 87 % compared to the TH enzyme activity in the contralateral striatum, and the TH enzyme activity was completely normalized in rats treated with the clone 877 DNA encapsulated within the TfR-PIL (Table 2). In contrast, the intravenous administration of clone 877 DNA encapsulated in the mIgG2a-PIL resulted in no increase in the TH enzyme activity in the ipsilateral striatum (Table 2). The TH enzyme activity in the cortex was only 2% of the contralateral striatum and there was no change in the cortical TH enzyme activity in the lesioned animals treated with the TfRMAb-PIL (Table 2).

The level of immunoreactive TH was measured by immunocytochemistry and there was a complete loss of immunoreactive TH in the dorsal striatum ipsilateral to the neurotoxin injection, although residual immunoreactive TH was detected in the ventral striatum and olfactory tubercle below the anterior commissure in some rats (Figure 4, panels B, D, F). The administration of clone 877 encapsulated within the mIgG2a-PIL resulted in no increase in the level of immunoreactive TH in the striate body (Figure 4 D, E, F). However, the level of immunoreactive TH was normalized in the entire striate body three days following an intravenous injection of the clone 877 DNA encapsulated within the PIL targeted to brain with the TfRMAb (Figure 4, panels A, B, C).

Reversal of motor impairment following TH gene therapy

The apomorphine-induced contralateral rotation was quantified in rats three days after a single intravenous injection of the clone 877 TH expression plasmid encapsulated in either the TfRMAb-PIL or the mIgG2a-PIL. Contralateral rotations were counted for 20 minutes following the intraperitoneal injection of apomorphine. The total rotations in a 20 minute period are plotted in Figure 5. The mean rotations per minute (RPM) in the animals treated with the mIgG2a-PIL was 20 ± 5 (range 13-26 rpm per animal). In contrast, the animals treated with the clone 877 TH expression plasmid encapsulated within the TfRMAb-PIL demonstrated a 70% reduction in rotation to 6 ± 2 RPM (mean ± SD) with a range of 3-9 RPM per individual rat.

DISCUSSION

The results of these studies are consistent with the following conclusions. First, the striatal TH enzyme activity is normalized by the intravenous injection of non-viral TH expression plasmid that is encapsulated in the interior of a PIL targeted to neurons with a TfRMAb (Table 2). Second, the normalization of TH enzyme activity in the striatum is paralleled by the normalization of the level of immunoreactive TH protein, as measured by immunocytochemistry (Figure 4). Third, the specificity of the gene targeting system is strictly a function of the MAb, since the only difference between the TfRMAb-PIL and the mIgG2a-PIL is the receptor specificity of the targeting MAb conjugated to the pegylated liposome (Figure 2A). Fourth, there is no change in TH enzyme activity in cortex and no measurable immunoreactive cortical TH as determined by immunocytochemistry following the intravenous injection of the TH expression plasmid encapsulated in the TfRMAb-PIL (Table 2 and Figure 4). Fifth, there is a reversal of apomorphine-induced rotation behavior following intravenous TH gene therapy, with a 70% reduction of drug-induced rotation (Figure 5).

The normalization of TH enzyme activity in the ipsilateral striatum (Table 2) is consistent with the detection of the immunoreactive TH throughout the ipsilateral striatum in rats treated with the TfRMAb-PIL (Figure 4). For treatment of human PD, it may be necessary to transduce at least 50% of the striate body, since the threshold of PD symptoms occurs with a 50 % loss of nigral-striatal neurons (Booij et al, 2001). The transduction of 50-100 % of the striatum with a therapeutic gene is possible following delivery of the gene to brain via the trans-vascular route. All neurons in the brain are perfused by their own capillaries and the PIL carrying the therapeutic gene is delivered to the "doorstep" of virtually every neuron in the brain following transcytosis across the BBB in vivo (Pardridge, 2002). In contrast, only a small volume of the striatum on

the order of 0-1% is transduced following intra-cerebral injection of a viral vector (Kirik et al, 2002). The volume of striatum transduced by the viral vector can be increased so that up to 12 % of striatal neurons are transduced following the multiple striatal injections of virus in the rat (Kirik et al, 2002).

Transduction of cell lines with retroviral vectors carrying the TH gene increases TH enzyme activity to approximately 1600 pmol/hr/10^6 cells (Leff et al, 1998), which is comparable to the TH enzyme activity in COS-1 cells transduced with Lipofectamine and clone 877 (Figure 1), since 10^6 cells in culture is equivalent to about 1 mg_p. This level of TH enzyme activity is also generated in the human U87 glioma cells with the HIRMAb-PIL gene targeting system (Figure 3). Therefore, the level of transduction of a target cell with the PIL gene targeting system is comparable to either cationic lipids or viral vectors.

The cellular targeting of the PIL gene delivery system is a function only of the receptor specificity of the targeting MAb (Shi et al, 2001a; 2001b). The TfRMAb-PIL and the mIgG2a-PIL have identical formulations, except the mouse IgG2a isotype control antibody does not recognize the rat TfR. The TfRMAb triggers the receptor-mediated transcytosis of the PIL nanocontainer across the BBB in vivo. Once in brain interstitial space, the PIL undergoes receptor-mediated endocytosis into brain cells expressing the TfR. The TfR is widely expressed on neurons throughout the CNS (Mash et al, 1991). Once inside brain cells, the fusogenic lipids of the liposome cause release of the plasmid DNA, which is then transported into the nuclear compartment for expression of the trans-gene. The rapid intra-nuclear delivery of DNA to cells with the PIL gene targeting system has been demonstrated with confocal microscopy (Zhang et al, 2002b).

The TH enzyme activity is not increased in the cortex of the brain following intravenous administration of the TH expression plasmid encapsulated within the TfRMAb-PIL (Table 2, Figure 4). A similar finding is made in the human TH transgenic mouse model, which shows increased TH enzyme activity in the striatum, but only minor changes in the frontal cortex (Kaneda, et al, 1991). The inability to mount a measurable increase in cortical TH enzyme activity may be due to the absence of TH cofactor in this region (Shimoji et al, 1999). The TH enzyme has an obligatory cofactor, tetrahydrobiopterin (BH4), and the TH enzyme is not active in the absence of the local production of the BH4 cofactor (Hwang et al, 1998). Studies in knockout mice show the level of the TH protein, measured by either enzyme activity or Western blotting, is markedly diminished in animals lacking BH4 (Sumi-Ichinose et al, 2001). Both in situ hybridization and immunocytochemistry demonstrate that only monoaminergic neurons in the brain express the enzyme that is rate-limiting for BH4 synthesis, GTP-cyclohydrolase (GTPCH), and there is no measurable GTPCH produced in the cortex (Nagatsu et al, 1997; Hwang et al, 1998). Although the mRNA for GTPCH is very low in the striatum (Hirayama et al, 1993), the GTPCH protein is produced in nerve endings terminating in the striatum (Hwang et al, 1998). GTPCH is produced in cell bodies of multiple regions of the brain outside the striatum, particularly serotoninergic systems (Lentz and Kapatos, 1996). These neurons terminate in the striatum (Hwang et al, 1998), and enable the striatal production of BH4, a diffusible small molecule, within the lesioned striatum. Both the GTPCH and BH4 levels in the striatum of the 6-hyroxydopamine-lesioned rat are still a third of the concentrations in the non-lesioned animal (Levine et al, 1981). This residual GTPCH in the striatum provides the BH4 co-factor for the TH enzyme produced by the exogenous TH gene delivered to brain. In contrast, GTPCH is not produced in the neocortex, and it is not possible to increase TH enzyme activity in

this region of the brain by administration of the exogenous TH gene (Table 2). This is advantageous for the treatment of PD, since it is not desirable to augment dopamine production in cortical structures. TH enzyme activity is increased in rat glioma cells targeted with the TfRMAb-PIL or in human U87 glial cancer cells targeted with the HIRMAb-PIL (Figure 3). These findings are consistent with prior studies showing that both cultured rat glioma cells and cancer cell lines produce the GTPCH enzyme (Nussler et al, 1996; Vann et al, 2002). A modest increase in rat liver TH activity is observed in control rats (Table 1), and this is consistent with the expression of GTPCH in liver (Nagatsu et al, 1997). However, the TH enzyme activity in rat liver is 98% reduced compared to striatal TH enzyme activity (Tables 1-2).

The reversal of apomorphine-induced rotational behavior in the 6-hydroxydopamine lesioned rat is not seen in all studies involving transduction of the striatum with TH genes using viral vectors (Mandel et al, 1998; Kirik et al, 2002). The reversal of the aberrant rotational behavior in this model may require the transduction of a significant volume of striatum with the TH therapeutic gene, and this may not be possible with a single intra-striatal injection of a viral vector. In contrast, the transvascular delivery of the TH gene to brain enables the complete normalization of TH enzyme activity in the striatum (Table 2) and the normalization of the level of immunoreactive TH in the entire striate body (Figure 4). These findings are consistent with the reversal of apomorphine-induced rotational behavior following the single intravenous administration of the TH gene packaged in the PIL gene delivery system (Figure 5).

In summary, the injection of 6-hydroxydopamine in the medial forebrain bundle caused a nearly 90% decrease in ipsilateral TH enzyme activity and immunoreactive TH in the ipsilateral striatum. TH enzyme activity and immunoreactive TH in the striatum were normalized by a single intravenous injection of a non-viral TH expression plasmid encapsulated in the interior of

the PIL and targeted to neurons with a TfRMAb. These studies show that it is possible to achieve pharmacological effects in brain with gene therapy that is administered without craniotomy and without viral vectors. Since this form of gene therapy relies on episomal expression of the trans-gene without permanent integration of the host genome, repeated administration of the gene therapy is necessary (Zhang et al, 2002a). Exogenous genes driven by the SV40 promoter are expressed for more than a week after a single intravenous injection in the rat of the PIL gene delivery system. Gene expression measured either by enzyme activity or by Southern blotting of plasmid DNA decreases by 50% at 6 days after the single administration of the gene in the rat (Shi et al, 2001b). The persistence of exogenous gene expression in the brain may be enhanced with the use of specially designed promoters, which are less susceptible to promoter inactivation in vivo (Yew et al, 2001).

REFERENCES

Anton R, Kordower JH, Maidment NT, Manaster JS, Kane DJ, Rabizadeh S, Schueller SB, Yang J, Rabizadeh S, Edwards, RH, Markham CH, Bredesen DE (1994) Neural-targeted gene therapy for rodent and primate hemiparkinsonism. Exp Neurol 127: 207-218.

Armstrong RJE, Hurelbrink CB, Tyers P, Ratcliffe EL, Richards A, Dunnett SB, Rosser AE, Barker RA (2002) The potential for circuit reconstruction by expanded neural precursor cells explored through porcine xenografts in a rat model of Parkinson's disease. Exp Neurol 175: 98-111.

Boado RJ, Pardridge WM (1998) Ten nucleotide cis element in the 3'-untranslated region of GLUT1 glucose transporter mRNA increases gene expression via mRNA stabilization. Mol Brain Res 59: 109-113.

Booij J, Bergmans P, Winogrodzka A, Speelman JD, Wolters EC (2001) Imaging of dopamine transporters with [^{123}I]FP-CIT SPECT does not suggest a significant effect of age on the symptomatic threshold of disease in Parkinson's disease. Synapse 39: 101-108.

During MJ, Naegele JR, O'Malley KL, Geller AI (1994) Long-term behavioral recovery in Parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. Science 266: 1399-1403.

Dwyer KJ, Boado RJ, Pardridge WM (1996) Cis-element/cytoplasmic protein interactions within the 3'-untranslated region of GLUT1 glucose transporter mRNA. J Neurochem 66: 449-458.

Hirayama K, Lentz SI, Kapatos G (1993) Tetrahydrobiopterin cofactor biosynthesis: GTP cyclohydrolase I mRNA expression in rat brain and superior cervical ganglia. J Neurochem 61: 1006-1014.

Horellou P, Guibert B, leviel V, Mallet J (1989) Retrovial transfer of a human tyrosine hydroxylase cDNA in various cell lines: regulated release of dopamine in mouse anterior pituitary AtT-20 cells. Proc Natl Acad Sci USA 86: 7233-7237.

Huwyler J, Wu D, Pardridge WM (1996) Brain drug delivery of small molecules using immunoliposomes. Proc. Natl Acad Sci USA 93: 14164-14169.

Hwang O, Baker H, Gross S, Joh TH (1998) Localization of GTP cyclohydrolase in monaminergic but not nitric oxide-producing cells. Synapse 28: 140-153.

Jefferies WA, Brandon MR, Hunt SV, Williams AF, Gatters KC, Mason DY (1984) Transferrin receptor on endothelium of brain capillaries. Nature 312: 162-163.

Kaneda N, Sasaoka T, Kobayashi K, Kiuchi K, Nagatsu I, Kurosawa Y, Fujita K, Yokoyama M, Nomura T, Katsuki M, Nagatsu T (1991) Tissue-specific and high-level expression of the human tyrosine hydroxylase gene in transgenic mice. Neuron 6: 583-594.

Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ (1994) Long-term gene expression and phenotypic correction using adeno- associated virus vectors in the mammalian brain. Nat Genet 8:148-154.

Kirik D, Georgievska B, Burger C, Winkler C, Muzyczka N, Mandel RJ, Bjorklund A (2002) Reversal of motor impairments in parkinsonian rats by continous intrastriatal delivery of L-dopa using rAAV-mediated gene transfer. Proc Natl Acad Sci USA 99: 4708-4713.

Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen E-Y, Palfi S, Roitberg BZ, Brown WD, Holden JE, Pyzalski R, Taylor MD, Carvey P, Ling Z, Trono D, Hantraye P, Deglon N, Aebischer P (2000) Neurodegeneration prevented by lentiviral vector deliver of GDNF in primate models of Parkinson's disease. Science 290: 767-773.

Leff SE, Rendahl KG, Spratt SK, Kang UJ, Mandel RJ (1998) In vivo L-DOPA production by genetically modified primary rat fibroblast or 9L gliosarcoma cell grafts via coexpression of GTPcyclohydrolase I with tyrosine hydroxylase. Exp Neurol 154: 249-264.

Lentz SI, Kapatos G (1996) Tetrahydrobiopterin biosynthesis in the rat brain: heterogeneity of GTP cyclohydrolase I mRNA expression in monoamine-containing neurons. Neurochem Int 28:569-582.

Leone P, McPhee SWJ, Janson CG, Davidson BL, Freese A, During MJ (2000) Multi-site partitioned delivery of human tyrosine hydroxylase gene with phenotypic recovery in Parkinsonian rats. Neuroreport 2: 1145-1151.

Levine RA, Miller LP, Lovenberg W (1981) Tetrahydrobiopterin in striatum: localization in dopamine nerve terminals and role in catecholamine synthesis. Science 214: 919-921.

Mandel RJ, Rendahl KG, Spratt SK, Snyder RO, Cohen LK, Leff SE (1998) Characterization of intrastriatal recombinant adeno-associated virus-mediated gene transfer of human tyrosine hydroxylase and human GTP-cyclohydrolase I in rat model of Parkinson's disease. J Neurosci 18(11): 4271-4284.

Mandel RJ, Rendahl KG, Snyder RO, Leff SE (1999) Progress in direct striatal delivery of L-dopa via gene therapy for treatment of Parkinson's disease using recombinant adeno-associated viral vectors. Exp Neurol 159:47-64.

Mash DC, Pablo J, Buck BE, Sanchez-Ramos J, Weiner WJ (1991) Distribution and number of transferrin receptors in Parkinson's disease and in MPTP-treated mice. Exp Neurol 114: 73-81.

Meshul CK, Kamel D, Moore C, Kay TS, Krentz L (2002) Nicotine alters striatal glutamate function and decreases the apomorphine-induced contralateral rotations in 6-OHDA-lesioned rats. Exp Neurol 175: 257-274.

Monnard P-A, Oberholzer T, Luisi P (1997) Entrapment of nucleic acids in liposomes. Biochim Biophys Acta 1329: 39-50.

Mouradian M.M. and Chase T.N. (1997) Gene therapy for Parkinson's disease: an approach to the prevention or palliation of levodopa-associated motor complications. Exp Neurol 144: 51-57

Nagatsu I, Arai R, Sakai M, Yamawaki Y, Takeuchi T, Karasawa N, Nagatsu T (1997) Immunohistochemical colocalization of GTP cyclohydrolase I in the nigrostriatal system with tyrosine hydroxylase. Neurosci Lett 224: 185-188.

Nussler AK, Liu Z-Z, Hatakeyama K, Geller DA, Billiar TR, Morris SM Jr (1996) A cohort of supporting metabolic enzymes is coinduced with nitric oxide synthase in human tumor cell lines. Cancer Lett 103: 79-84.

Pardridge W (2002) Drug and gene targeting to the brain with molecular Trojan horses. Nature Rev Drug Disc 1: 131-139.

Pardridge WM, Buciak JL, Yoshikama, T (1992) Transport of recombinant CD4 through the rat blood-brain barrier in vivo. J Pharmacol Exp Ther 261: 1175-1180.

Pardridge WM, Eisenberg J, Yang J (1987) Human blood-brain barrier transferrin receptor. Metabolism 36: 892-895.

Reinhard JF, Smith GK, Nichol CA (1986) A rapid and sensitive assay for tyrosine-3-monooxygenase based upon the release of $3H_2O$ and adsorption of [^{3}H]-tyrosine by charcoal. Life Sci 39: 2185-2189.

Shi N, Pardridge WM (2000) Noninvasive gene targeting to the brain. Proc. Natl Acad Sci USA 97: 7567-7572.

Shi N, Zhang Y, Boado RJ, Zhu C, Pardridge WM (2001a) Brain-specific expression of an exogenous gene following intravenous administration. Proc Natl Acad Sci USA 98: 12754-12759.

Shi N, Boado RJ, Pardridge WM (2001b) Receptor-mediated gene targeting to tissues in the rat in vivo. Pharm Res, 18: 1091-1095.

Shimoji M, Hirayama K, Hyland K, Kapatos G (1999) GTP cyclohydrolase I gene expression in the brains of male and female hph-1 mice. J Neurochem 72: 757-764.

Shusta EV, Boado RJ, Pardridge WM (2002) Vascular proteomics and subtractive antibody expression cloning. Molec Cellular Proteomics 1: 75-82.

Sumi-Ichinose C, Urano F, Kuroda R, Ohye T, Kojima M, Tazawa M, Shiraishi H, Yasumichi H, Nagatsu T, Nomura T, Ichinose H. (2001) Catecholamines and serotonin are differently regulated by tetrahydrobiopterin. J Biol Chem 276: 41150-41160.

Vann LR, Payne SG, Edsall LC, Twitty S, Spiegel S, Milstien S (2002) Involvement of sphingosine kinase in TNF-α-stimulated tetrahydrobiopterin biosynthesis in C6 glioma cells. J Biol Chem 277: 12649-12656.

Yew NS, Prybylska M, Ziegler RJ, Liu D, Cheng SH (2001) High and sustained transgene expression in vivo from plasmid vectors containing a hybrid ubiquitin promoter. Mol Ther 4: 75-82.

Zhang Y, Zhu C, Pardridge WM (2002a) Antisense gene therapy of brain cancer with an artificial virus gene delivery system. Mol Ther 6: 67-72.

Zhang Y, Lee HJ, Boado RJ, Pardridge WM (2002b) Receptor-mediated delivery of an antisense gene to human brain cancer cells. J Gene Med 4: 183-194.

Table 1. Tyrosine hydroxylase (TH) activity in liver after intravenous injection of clone 877 plasmid DNA encapsulated in the TfRMAb-PIL in control rats

Days post-administration	TH activity (pmol L-Dopa/hour/mg_p)
0	3.0 ± 0.3
1	18.2 ± 1.8
2	33.2 ± 2.7
3	103.7 ± 9.1

Mean ± S.D. (n= 3 rats per time point).

Table 2. Tyrosine hydroxylase (TH) activity in rat brain lesioned with 6-hydroxydopamine at 3 days after intravenous injection of clone 877 plasmid DNA encapsulated in either TfRMAb-PIL or mIgG2a-PIL

region	TH activity (pmol L-Dopa/hr/mg_p)	
	TfRMAb-PIL	**mIgG2a-PIL**
ipsilateral striatum	5486 ± 899 *	738 ± 179
contralateral striatum	5875 ± 550 *	5101 ± 443 *
ipsilateral cortex	159 ± 27	101 ± 7
contralateral cortex	121 ± 39	108 ± 51

Mean ± SD (n=5 rats each group)

* $p<0.01$ difference from ipsilateral striatum treated with mIgG2a-PIL (1-way ANOVA, Bonferroni correction)

Figure 1. Tyrosine hydroxylase (TH) activity in either COS-1 cells or C6 rat glioma cells transfected with one of 5 different expression plasmids in cell culture with Lipofectamine. Clones 883 and 908 are derived from pCEP4, which contains an EBNA1/oriP trans/cis element, and clones 877 and 878 are derived from pGL2, which lacks the EBNA1/oriP elements. Clones 877 and 908 contain a 200 base pair cis-element taken from the 3'-untranslated region (UTR) of the Glut1 glucose transporter mRNA, and this cis element causes stabilization of the transcript. Clone 922 is a pGL3 luciferase expression plasmid and this clone produced no measurable TH enzyme activity in either cell line (control=ctrl). Data mean ± SE (n=4 dishes per point).

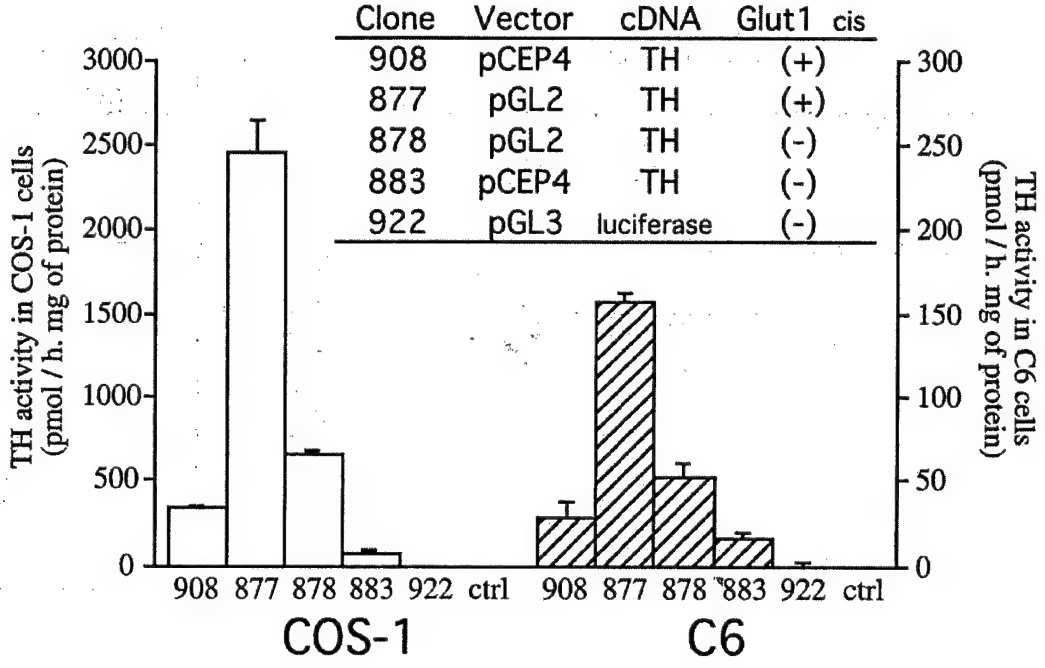

Figure 2(A). Plasmid DNA encapsulated in the interior of the pegylated immunoliposome (PIL) with a receptor (R)-specific targeting monoclonal antibody (MAb). The targeting MAb is conjugated to 1-2% of the polyethyleneglycol (PEG) strands that project from the surface of the liposome. There are about 2000 strands of 2000 Dalton PEG conjugated to the liposome surface. The PEG strands inhibit uptake of the PIL by the reticuloendothelial systems in vivo and prolong the blood residence time of the PIL in vivo (Shi et al, 2000). The tyrosine hyrdoxylase (TH) gene is driven by an SV40 promoter (pro) and contains a cis-stabilizing element in a 3'-untranslated region (UTR). (B). Transmission electron microscopy of a PIL. The mouse IgG molecule tethered to the tips of the 2000-Dalton polyethylene glycol (PEG) are bound by a conjugate of 10 nm gold and an anti-mouse secondary antibody. The position of the gold particles illustrates the relationship of the PEG extended MAb and the liposome. Magnification bar = 20 nm.

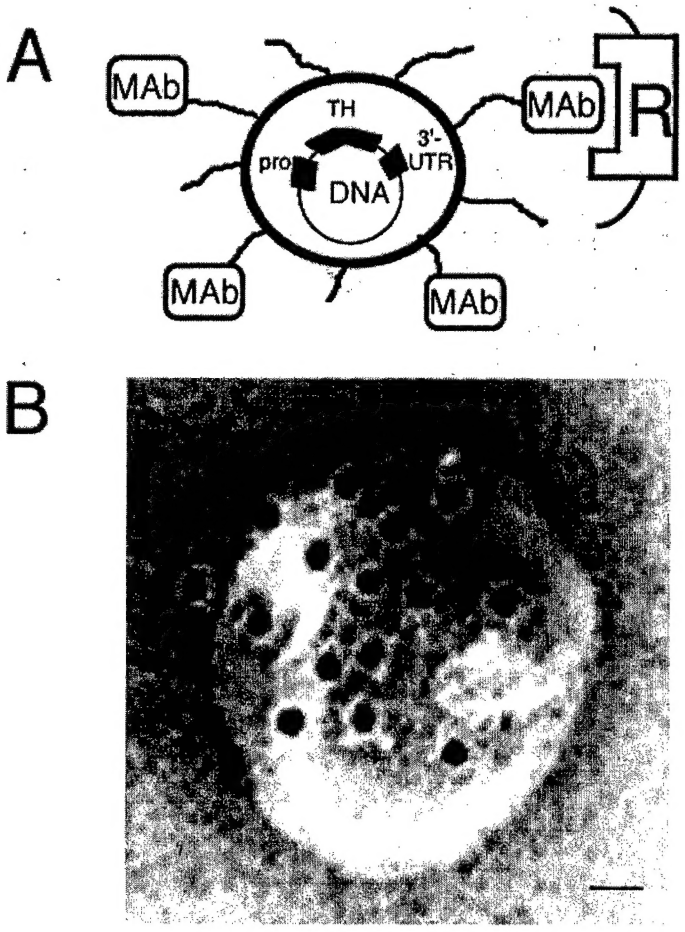

Figure 3. Tyrosine hydroxylase activity is plotted relative to the days of incubation in cell culture of either rat RG-2 cells or human U87 glioma cells exposed to the clone 877 plasmid DNA encapsulated in the PIL. The PIL was targeted to the RG-2 cells with the OX26 murine MAb to the rat transferrin receptor (rTfR) and the PIL was targeted to human cells with the 83-14 murine MAb to the human insulin receptor (HIR). Data are mean ± SE (n=4 dishes per point).

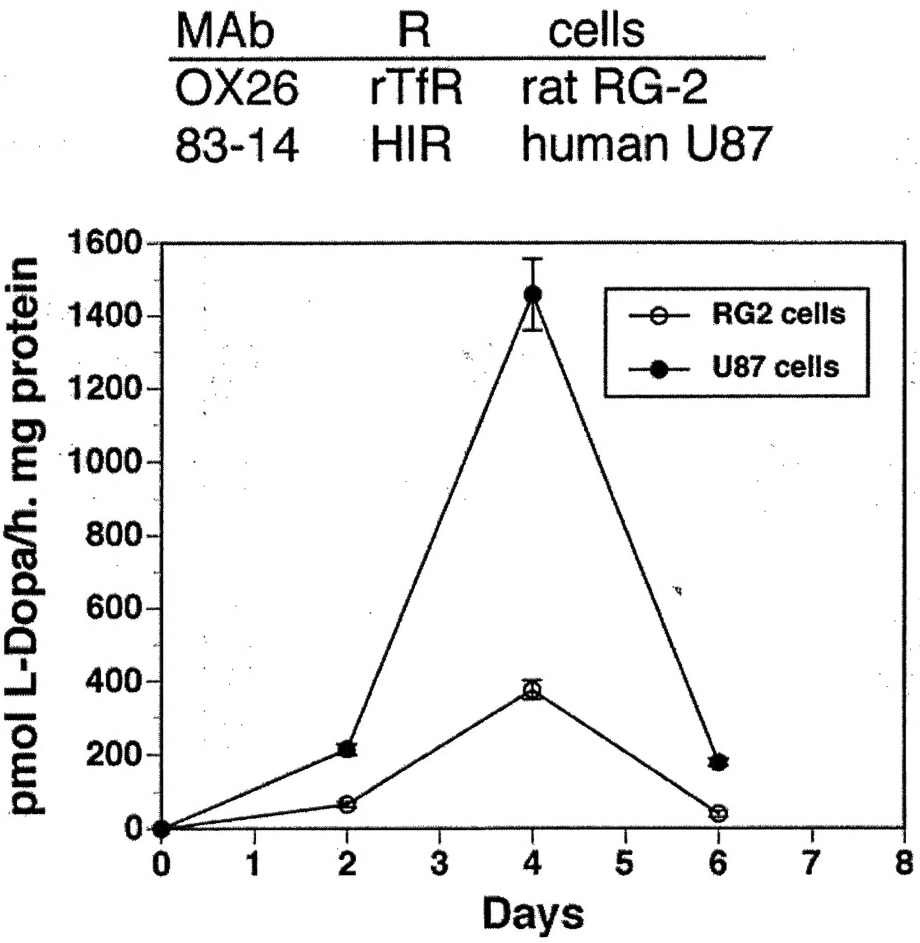

Figure 4. Tyrosine hydroxylase immunocytochemistry of rat brain removed 72 hours after a single intravenous injection of 10 ug per rat of clone 877 plasmid DNA encapsulated in PIL targeted with either the TfRMAb (Panels A, B, C) or with the mouse IgG2a isotype control (Panels D, E, F). Coronal sections are shown for 3 different rats from each of the two treatment groups. The 6-hydroxydopamine was injected in the medial forebrain bundle of the right hemisphere, which corresponds to right side of the figure. Panels G, H, I are coronal sections of brain stained with hematoxylin. Panels G, H, and I correspond to panels A, E, and F, respectively. Immunoreactive TH is completely abolished in both the caudal and ventral striatum in the rat shown in panel E, whereas there is residual immunoreactive TH in the ventral striatum and olfactory tubercle in the rats shown in panels D and F.

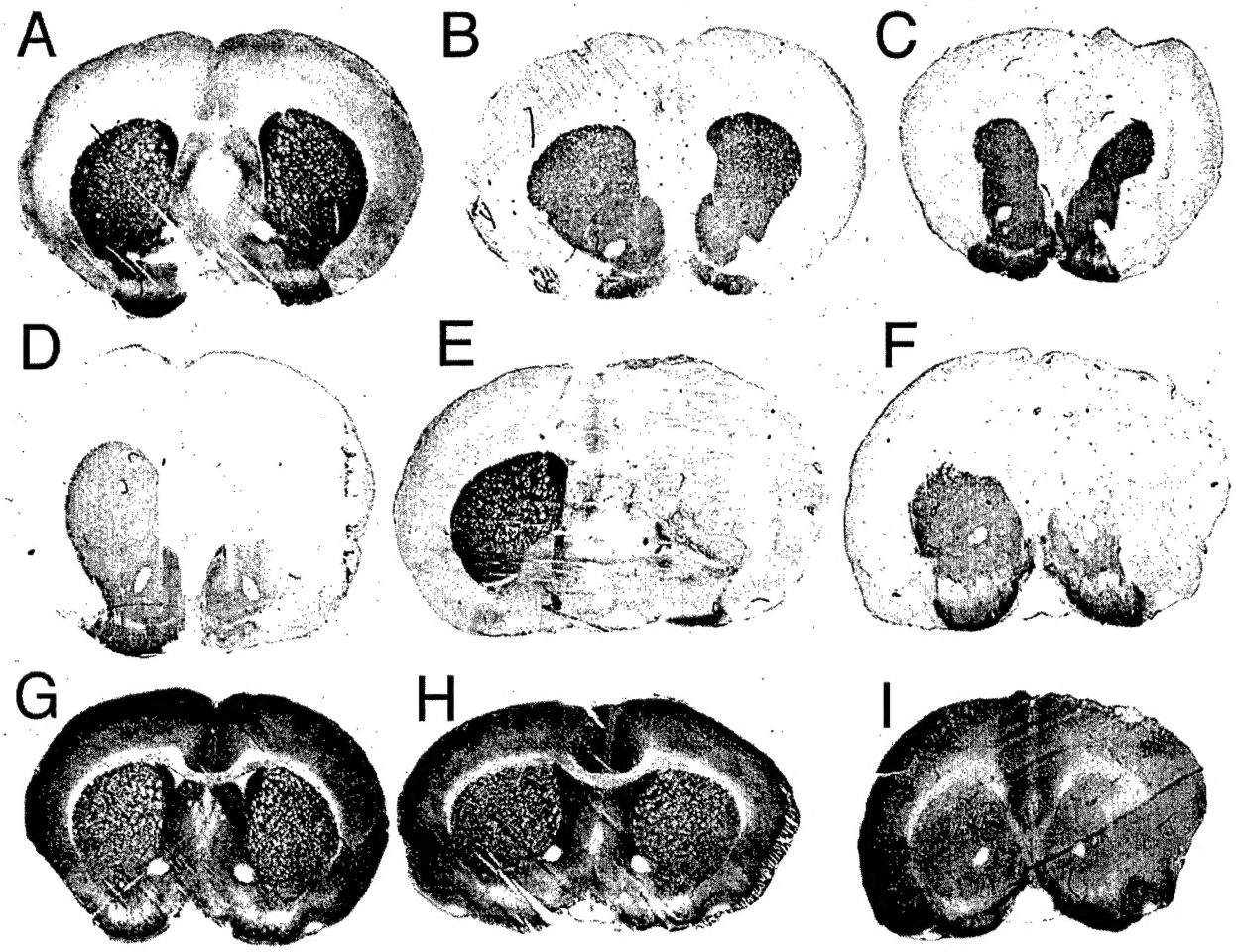

Figure 5. Total apomorphine-induced rotations over a 20 minute period measured in rats at three days after a single intravenous injection of 10 microgram per rat of clone 877 plasmid DNA encapsulated in a PIL targeted with either the TfRMAb with the mouse IgG2a isotype control antibody. The rotations per minute (RPM) in the animals treated with the mIgG2a-PIL was 20 ± 5 and the RPM in animals treated with the TfRMAb-PIL was 6 ± 2 (mean ± SD). The difference in rotation between the 2 groups was significant at the $p < 0.005$ level (Student's t-test).